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Review

Protonmotive cooperativity in cytochrome *c* oxidase

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Abstract

Cooperative linkage of solute binding at separate binding sites in allosteric proteins is an important functional attribute of soluble and membrane bound hemoproteins. Analysis of proton/electron coupling at the four redox centers, i.e. Cu_A, heme *a*, heme a_3 and Cu_B, in the purified bovine cytochrome *c* oxidase in the unliganded, CO-liganded and CN-liganded states is presented. These studies are based on direct measurement of scalar proton translocation associated with oxido-reduction of the metal centers and pH dependence of the midpoint potential of the redox centers.

Heme *a* (and Cu_A) exhibits a cooperative proton/electron linkage (Bohr effect). Bohr effect seems also to be associated with the oxygen-reduction chemistry at the heme a_3–Cu_B binuclear center. Data on electron transfer in cytochrome *c* oxidase are also presented, which, together with structural data, provide evidence showing the occurrence of direct electron transfer from Cu_A to the binuclear center in addition to electron transfer via heme *a*.

A survey of structural and functional data showing the essential role of cooperative proton/electron linkage at heme *a* in the proton pump of cytochrome *c* oxidase is presented. On the basis of this and related functional and structural information, variants for cooperative mechanisms in the proton pump of the oxidase are examined.

Keywords: Cytochrome *c* oxidase; Proton pumping; Cooperativity

1. Introduction

In coupling membranes of prokaryotes and eukaryotes, the energy-transfer redox complexes of the respiratory chain convert the free energy, made available by downhill electron transfer, into a transmembrane electrochemical proton gradient (protonmotive force, PMF). PMF is, in turn, utilized to drive ATP synthesis from ADP and P_i and ion transport [1,2].

In the sixties, Mitchell proposed the protonmotive activity of redox complexes as a direct consequence of primary redox catalysis (protonmotive redox loops) [1]. This direct formalism attributed to the apoproteins only a passive role to hold the redox prosthetic groups in the proper orientation in the membrane and, possibly, to provide proton conduction pathways (proton wells) connecting them to the aqueous phases [3]. Work that followed has produced detailed models of direct redox protonmotive catalysis, as for example the quinone cycle of *bc*-type complexes [4]. At the same time, evidence became, on the other hand, available of protonmotive cooperative interactions in redox components of the respiratory chain [2]. Cytochromes *b* and *a* [5] and iron-sulfur proteins [6] exhibit pH dependence of the redox potential, revealing a linkage between the oxido-reduction of the metal centers and proton binding/dissociation in these electron transfer proteins [7].

Based on the principles of cooperative linkage of solute binding at separate sites in allosteric proteins [8], in particular hemoglobin [9], Papa et al. [10,11] proposed in the seventies a model based on cooperative redox-linked p*K* shifts in electron transfer proteins (redox Bohr effect) for proton pumping in respiratory chains (vectorial Bohr mechanism). It was postulated that reduction of the metal

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prosthetic center in a redox enzyme in the membrane results in the p*K* increase of a residue in the protein in protonic connection with the inner (N) side of the membrane with proton uptake from this space, and oxidation of the metal in the decrease of the p*K* of this group, or another in protonic connection with the first, with proton release in the outer (P) space [11]. This principle now seems to be widely incorporated in recent models of redox-linked proton translocation [12–16]. It is, in fact, feasible that cooperative protonmotive events operate in proton pumps in series or in parallel with direct protonmotive catalytic steps.

Cytochrome *c* oxidase (COX), as well as other members or the superfamily of heme-copper oxidases, conserves the free energy made available in the reduction of O_2 to H_2O by ferrocytochrome *c* as a PMF [17,18]. Generation of PMF first results from the consumption of protons from the N space in the reduction of O_2 to H_2O by ferrocytochrome *c* located at the P side of the membrane [11,17], as originally postulated by Mitchell [1,3]. In addition to this, COX displays a net proton pumping activity from the N to the P space, coupled to electron flow from ferrocytochrome *c* to O_2 [17,19]. Although this process is a matter of intensive investigation in many laboratories, its detailed molecular mechanism is not yet fully understood. Cytochrome *c* oxidase has four redox centers: a binuclear Cu_A center, titrating as one electron redox entity, which is bound to subunit II, heme *a*, heme a_3 and Cu_B; these all bound to subunit I [20]. Cytochrome *c* delivers electrons to Cu_A; heme a_3 and Cu_B constitute the binuclear center where O_2 is reduced to H_2O. Heme *a* mediates electron transfer from Cu_A to the binuclear center [20]. At present, two general models are favoured in exploring the proton pump of heme-copper oxidases. One is a "direct" coupling model confined at the heme a_3–Cu_B binuclear center in which the protonmotive steps of O_2 reduction to H_2O are directly associated with transmembrane proton translocation [16,21]. The other is an "indirect" coupling model in which the proton pump is cooperatively coupled to electron transfer at the heme *a* (and Cu_A) center of the oxidase [22–26]. This separation may, however, not be so definite [23,27].

In this paper, a description of cooperative proton/electron linkage at the redox centers of cytochrome *c* oxidase is presented. The role of cooperative linkage at heme *a* in proton pumping, in particular, will be examined.

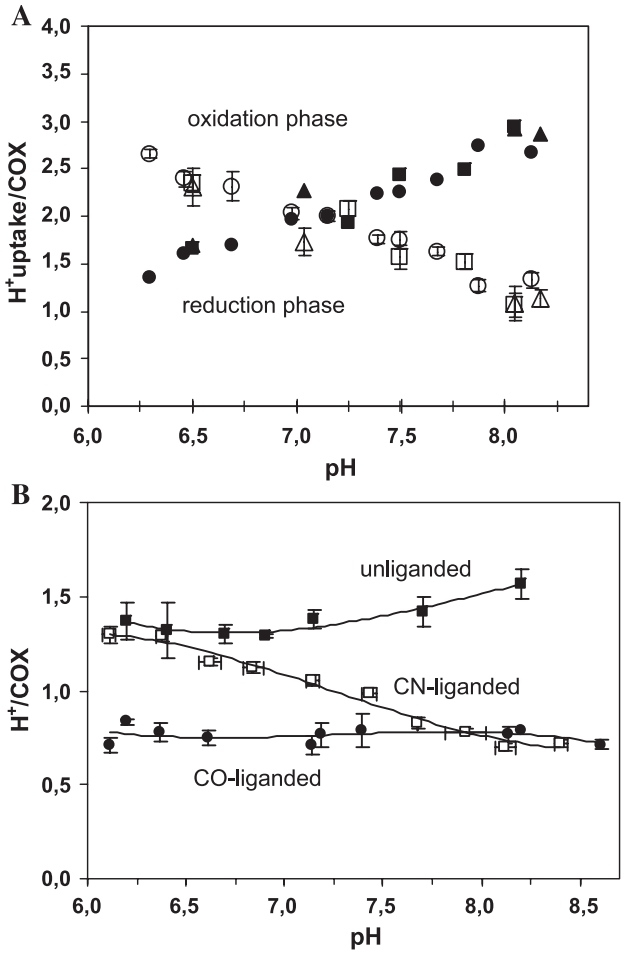

Fig. 1. pH dependence of proton transfer associated with oxido-reduction of metal centers in purified bovine heart cytochrome *c* oxidase. (A) pH dependence of the H^+/COX ratios for proton uptake associated with aerobic oxidation and re-reduction of metal centers in COX. For experimental details, see Ref. [28]. Open symbols refer to the H^+ uptake/COX ratios associated with oxidation of fully reduced COX by addition of O_2. Closed symbols are H^+/COX ratios for proton uptake in the re-reduction of the oxidised enzyme. (B) pH dependence of proton release/uptake associated with oxido-reduction of metal centers induced by ferricyanide/ferrocyanide in anaerobic soluble bovine heart cytochrome *c* oxidase. Black squares: H^+/COX ratios associated with oxido-reduction of hemes $a+a_3$, Cu_A and Cu_B in the unliganded COX. Empty squares: H^+/COX ratios associated with oxido-reduction of heme *a*, Cu_A and Cu_B in the CN-liganded COX, heme a_3 clamped in the oxidized state. Black circles: H^+/COX ratios associated with oxido-reduction of heme *a* and Cu_A in the CO-liganded COX, heme a_3 and Cu_B clamped in the reduced state. For details, see Refs. [28,29].

2. Proton/electron coupling at the redox centers

Fig. 1 summarizes the results of direct measurements of proton transfer reactions associated with oxido-reduction of metal centers in purified bovine heart COX. Fig. 1A shows that the expected proton consumption associated with oxidation of the reduced four metal centers (Cu_A^{1+}, a^{2+}, a_3^{2+}, Cu_B^{1+}) by oxygen decreases with pH, going from around 3 H^+/COX at pH 6.2–6.3 to ~1.2 H^+/COX at pH 8.0–8.5. Re-reduction of metal centers is associated with further proton uptake, which increases as the pH is raised. The sum of the H^+ uptake measured in the oxidation and reduction phase, respectively, amounts to the overall consumption of 4 H^+ in the reduction of O_2 to $2H_2O$ [28]. These results show that the expected stoichiometric proton consumption of $4H^+$ in the reduction of O_2 to $2H_2O$ is differently associated, depending on

the actual pH, with the oxidation and reduction phase of COX (Fig. 2A).

Fig. 1B shows the H^+/COX ratios for proton release associated with anaerobic oxidation of reduced metal centers by ferricyanide in the reduced unliganded COX, CN-liganded COX and CO-liganded COX. In all the three conditions, the H^+ release is completely reversed upon re-reduction of the metal centers. Anaerobic ferricyanide oxidation of the fully reduced unliganded oxidase gives H^+/COX release ratios which vary between 1.4 at pH 6.2 and 1.6 at pH 8.2 (bH^+ in Eq. (1)). Anaerobic ferricyanide oxidation of the CN-liganded COX, in which heme a_3 is blocked in the oxidised state, thus leaving Cu_A, heme a and Cu_B to undergo oxido-reduction cycle, gives H^+/COX release ratios which vary from 1.35 at pH 6.2–6.3 to 0.8 at pH 8.2 ($b'H^+$ in Eq. (2)). This H^+/COX release is at pHs below 7.5, higher than the H^+/COX ratio for proton release associated with ferricyanide oxidation of Cu_A^+ and heme a^{2+} in the CO-liganded oxidase in which Cu_B and heme a_3 are blocked in the reduced state. Under the latter conditions, the H^+/COX for proton release/uptake associated with oxido-reduction of Cu_A and heme a varies between 0.65 and 0.90 in the pH range 6.0–8.5 (nH^+ in Eq. (3)) (Fig. 1B, see Ref. [29]). Oxido-reduction of both Cu_A and heme a in the CO-

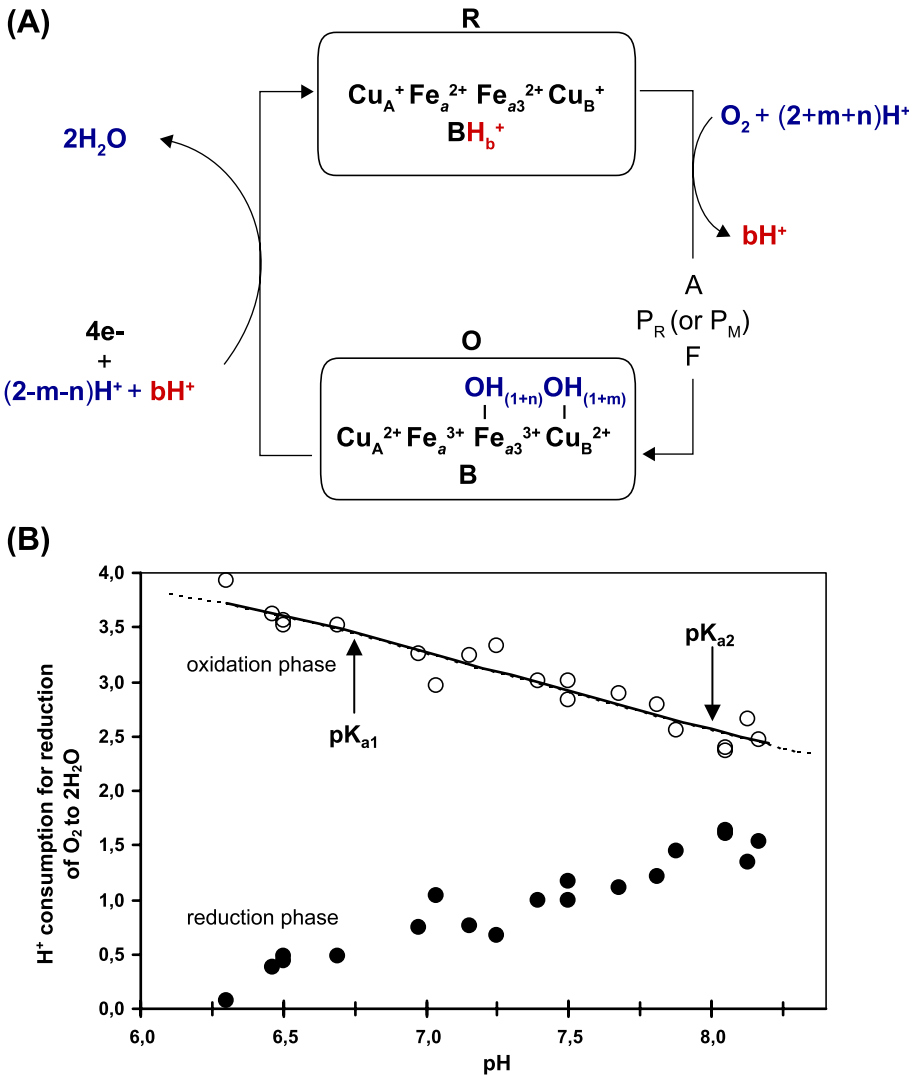

Fig. 2. (A) Schematic drawing of the protonation and ligation states associated with the oxido-reduction cycle of cytochrome *c* oxidase under aerobic conditions. Bohr protons and chemical protons are shown in red and blue, respectively. For further explanation, see text. (B) pH dependence of proton consumption in the reduction of O_2 to $2H_2O$ by COX. Open symbols are obtained by adding to the H^+ uptake/COX ratios measured in the conversion of COX from the fully reduced to fully oxidised state (Fig. 1A), the H^+ release/COX ratios measured in the anaerobic oxidation by ferricyanide at the corresponding pHs shown in Fig. 1B. The line represents the best fit of the corrected values for the proton uptake associated with oxidation of fully reduced cytochrome *c* oxidase by O_2, obtained with the contribution of two protolytic groups with p*K*s of 6.75 and 8.0, respectively. Closed circles are obtained by subtracting from the H^+ uptake/COX ratios measured in the conversion of the COX from the fully oxidised to the fully reduced state (Fig. 1A), the H^+/COX ratios measured for proton uptake in the reduction of COX by ferrocyanide at the corresponding pHs (Fig. 1B). For experimental details, see Ref. [28]. Reproduced with permission from Ref. [28].

COX has, in fact, been shown to be cooperatively linked to p*K* shifts in a common network of acid/base groups [29,30].

$$[Cu_A^+ a^{2+} a_3^{2+} Cu_B^+] \cdot (bH^+) + 4Fe(CN)_6^{3-} \rightarrow [Cu_A^{2+} a^{3+} a_3^{3+} Cu_B^{2+}] + bH^+ + 4Fe(CN)_6^{4-} \quad (1)$$

$$[Cu_A^+ a^{2+} a_3^{3+} CNCu_B^+] \cdot (b' H^+) + 3Fe(CN)_6^{3-} \rightarrow [Cu_A^{2+} a^{3+} a_3^{3+} CNCu_B^{2+}] + b' H^+ + 3Fe(CN)_6^{4-} \quad (2)$$

$$[Cu_A^+ a^{2+} a_3^{2+} COCu_B^+] \cdot (bH^+) + 2Fe(CN)_6^{3-} \rightarrow [Cu_A^{2+} a^{3+} a_3^{2+} COCu_B^+] \cdot (b-n)H^+ + nH^+ + 2Fe(CN)_6^{4-} \quad (3)$$

The overall H^+ release/uptake observed upon anaerobic ferri/ferro-cyanide oxido-reduction of unliganded COX represents the net result of Bohr protons linked to oxido-reduction of the metal centers and ligand exchange at the binuclear center. The H^+/COX ratio measured under these conditions, which at acidic pH corresponds to that observed for proton uptake in the re-reduction of the aerobically oxidised COX, does not increase at alkaline pH as observed in the aerobic cycle, where it reaches at pH around 8.0 the value of ≈ 2.8. This can be ascribed to suppression of OH^-/H_2O exchange at the binuclear site by chloride binding to the oxidised enzyme [31]. It should be noted that 150 mM KCl was present in the reaction medium used in the ferri/ferro-cyanide cycle [28]. Under these conditions, the H^+/COX ratio could represent the net result of Bohr protons linked to oxido-reduction of the COX metal centers. Of these 0.65–0.9 H^+/COX are associated with oxido-reduction of Cu_A and heme *a*; the remaining H^+/COX exchange pertains to Bohr protons associated with oxido-reduction of the binuclear center. In the CN-liganded oxidase the inhibitor occupies the binuclear site, thus preventing exchange of other ligands [31]. The H^+/COX exchange, in the CN-liganded COX, thus represents Bohr protons linked to oxido-reduction of Cu_A, heme *a* and Cu_B. From the H^+/COX ratios observed under the three conditions of the ferri-/ferro-cyanide oxido-reduction cycle, it can be tentatively calculated that the H^+/COX ratio for Bohr protons associated with Cu_B oxido/reduction varies from 0.6 at pH 6.0 to 0 at pH 8, with overall $pK_{ox} = 5.6$ and $pK_{red} = 6.7$ of the group(s) linked to this metal. An H^+/COX ratio for Bohr protons associated with oxido-reduction of heme a_3, varying from 0.1 at pH 6.0 to 0.9 at pH 8.0, with a $pK_{ox} = 7.5$ and $pK_{red} > 12$, can also be estimated.

The addition to the proton uptake, observed in the aerobic oxidative phase of reduced COX (Fig. 1A), of the proton release elicited by anaerobic ferricyanide oxidation of unliganded COX (Fig. 1B), gives H^+/COX ratios for proton consumption in this phase which vary from ≈ 4 at pH 6.3 to ≈ 2.5 at pH 8.2 (Fig. 2, panel B, open circles) [28]. Subtraction from the proton consumption, observed in the reductive phase of the aerobic experiments (Fig. 1A), of the H^+ uptake promoted by ferrocyanide COX reduction (Fig. 1B), gives H^+/COX ratios which increase with pH from 0 at pH 6.3 to ≈ 1.5 at pH 8.2 (Fig. 2, closed circles), thus complementing to 4 the proton consumption measured in the oxidative phase. The experimental points obtained for the proton consumption in the oxidation phase can be best fitted by an equation based on two p*K*s of 6.7 and 8.0, respectively [28]. It can be noted that p*K*s in this range have been estimated for water associated with the binuclear center (cf. Refs. [32,33]).

The p*K* shifts linked to oxido-reduction of both Cu_A and heme *a* result in pH dependence of E_m of both Cu_A and heme *a* in the CO-liganded COX of around 20 mV/pH unit [29]. It can be noted that, in the CO-COX, redox titrations of both heme *a* and CuA do not exhibit any detectable deviation from a regular Nerstian plot (Fig. 3B). On the contrary, redox titration of heme *a* in the CN-COX shows at acidic and neutral pH a significant deviation from a linear Nerstian plot, which tends, however, to return to normal at alkaline pHs (Fig. 3A) (see also Ref. [34]). This deviation is specific for heme *a*, in that Cu_A exhibits also in the CN-COX a linear Nerstian plot (Fig. 3A). This pattern is consistent with a pH-dependent negative cooperativity of heme *a* with Cu_B, resulting in a high E_m when Cu_B is oxidized and a low E_m when the latter is reduced (anti-cooperative interaction of heme *a* and Cu_B) [34].

These observations indicate that oxido-reduction of heme *a* shares protonmotive interaction with both Cu_A, on the cytochrome *c* side, and Cu_B on the oxygen side of the electron transfer pathway in the oxidase. Caution should, however, be used to extrapolate the patterns obtained in the CO and CN inhibited state to the unliganded oxidase in the turning over respiratory state, in which the population of reduced Cu_B and heme a_3 is likely to be negligible (cf. Ref. [27]). It should be added that in the unliganded oxidase a complex interaction occurs also between hemes *a* and a_3 [17].

3. Electron transfer pathways, proton pumping and role of heme *a*

It is generally thought that electron flow in the oxidase from Cu_A to the binuclear center proceeds only via heme *a*. Heme *a*, heme a_3 and Cu_B are bound in subunit I to conserved histidine residues of transmembrane helices II, X, VI and VII in regions extending towards the P surface (Fig. 4). The X-ray crystallographic structures of *Paracoccus denitrificans* [35] and bovine heart cytochrome *c* oxidase [36] show that the distances between the nearest Cu_A atom to heme *a* iron and to heme a_3 iron are quite similar, 19.5 and 22.1 Å, respectively. The propionate groups of both hemes *a* and a_3, which are perpendicular to the plane of the membrane, point towards and communicate through conserved residues in subunit I with the C-terminal

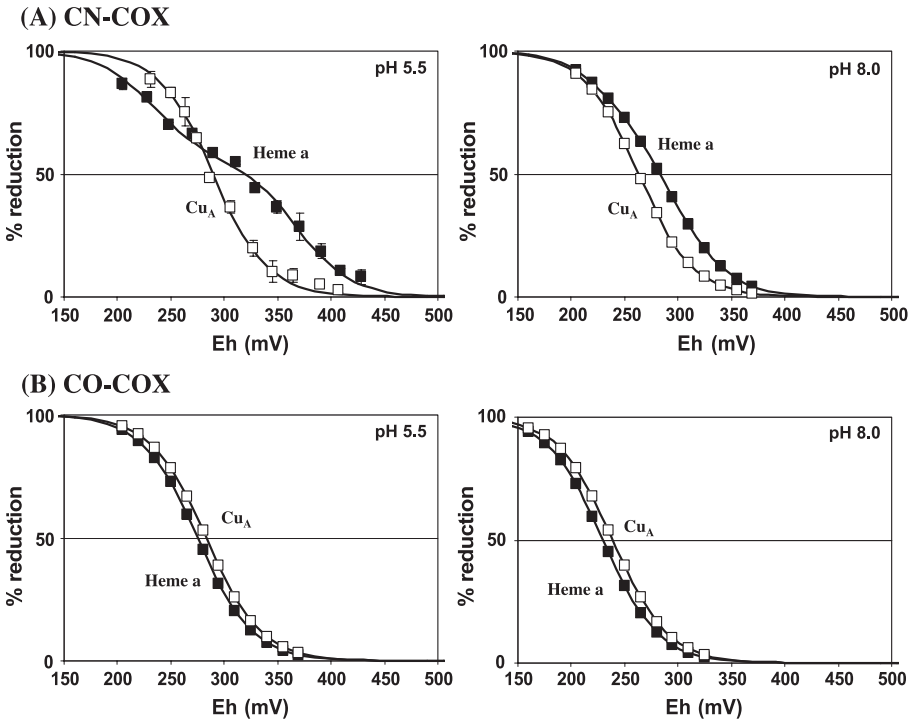

Fig. 3. Effect of pH on the redox titrations of heme *a* (black squares) and Cu_A (empty squares) in cyanide-liganded (A) and CO-liganded cytochrome *c* oxidase (B). Liganded COX was reduced under anaerobic conditions as described in Ref. [29] and stepwise oxidised and re-reduced by ferricyanide and dithionite; the levels of reduction were measured in the visible and near-infrared spectral region (500–900 nm) by diode array spectrophotometry. The E_h was measured under equilibrium conditions from the reduction level of cytochrome *c* (used as internal redox indicator/mediator). The experimental values for both heme *a* and Cu_A in the CO-liganded cytochrome oxidase and for Cu_A in the CN-liganded enzyme were fitted as simple Nernstian components ($n=1$). The distorted redox behaviour of heme *a* in the CN-liganded oxidase (at acidic pH) was fitted with a model-equation assuming a negative co-operative interaction between heme *a* and a redox interactant, according to the equation: $\%\text{reduction} = 100/(1+(10^{F/2.303RT(Eh-E1)})(1+e^{(F/RT(E2-Eh)})/(1+e^{(F/RT(E2-E'-Eh)}))$ where E_1 is the E_m of heme heme *a* when the interactant is oxidised, E_2 is the E_m of the interactant when heme *a* is oxidised, E' is the interactant energy; the values of E_1, E_2, E' were 354, 348, 100 mV at pH 5.5 and 291, 275, 26 mV at pH 8.0.

domain of subunit II holding the two copper atoms of the Cu_A center [35,36]. Residues have been located in subunit II and subunit I, providing a hydrogen bond/ion pair network which can serve as an electron transfer path between Cu_A and heme *a*. In this network, His 204, a Cu_A ligand in subunit II, is hydrogen-bonded to Arg 438 and Arg 439 (bovine numbering) in the loop XI–XII of subunit I; the latter are bonded to the propionate groups of heme *a*. Tsukihara et al. [36] have also identified in the bovine heart oxidase a hydrogen bond network involving the propionate groups of heme a_3, Arg 438 in the loop XI–XII and His 368 of subunit I, the latter coordinated by Mg^{2+} to Glu 198 in subunit II which is liganded to the lower Cu_A atom. This network, which can be seen to be conserved also in the X-ray structures of *P. denitrificans* [35] and *Rhodobacter sphaeroides* [37], could provide direct electron transfer from Cu_A to the heme a_3–Cu_B center [36] (Fig. 4). In all the oxidases so far analysed, the distance from the lower Cu_A to heme *a* Fe is ≈ 2.6 Å shorter than that to heme a_3 Fe. With this difference, the rate of electron transfer from Cu_A to heme *a* would intrinsically be much faster than to heme a_3. Sequence alignment based on X-ray crystallographic structure of the ba_3 cytochrome *c* oxidase from *Thermus thermophilus* with the bovine and *P. denitrificans* oxidase leads also to identify sequence motifs and structural arrangement of residues providing an additional electron transfer pathway leading directly from Cu_A to Cu_B [38]. It should be noted that Cu_A resides in a different subunit from that where the two hemes reside. The distances between Cu_A and the heme irons have been measured in the crystallized structures of COX. It is possible that in the oxidase turning over at the steady state, the contacts between subunit I and II and hence the distances between Cu_A and the heme irons can be slightly changed. In particular, the steady-state PMF might affect the subunit contacts.

The possibility that electron transfer from Cu_A to the binuclear center may take place also directly, thus bypassing heme *a*, seems to be supported by the reduction levels of the metal centers in liposome reconstituted bovine heart cytochrome *c* oxidase (COV). The results of this analysis show that both at level flow and uncoupled respiring steady-state, i.e. under conditions in which kinetic factors prevail and no significant effect is exerted by PMF, the reduction level of Cu_A is lower than that of heme *a* and is enhanced less than that of heme *a* by raising the rate of electron delivery to the oxidase (Fig. 5) [39].

Investigations on a mutant of the *P. denitrificans* cytochrome *c* oxidase, in which Arg 54 of subunit I was changed

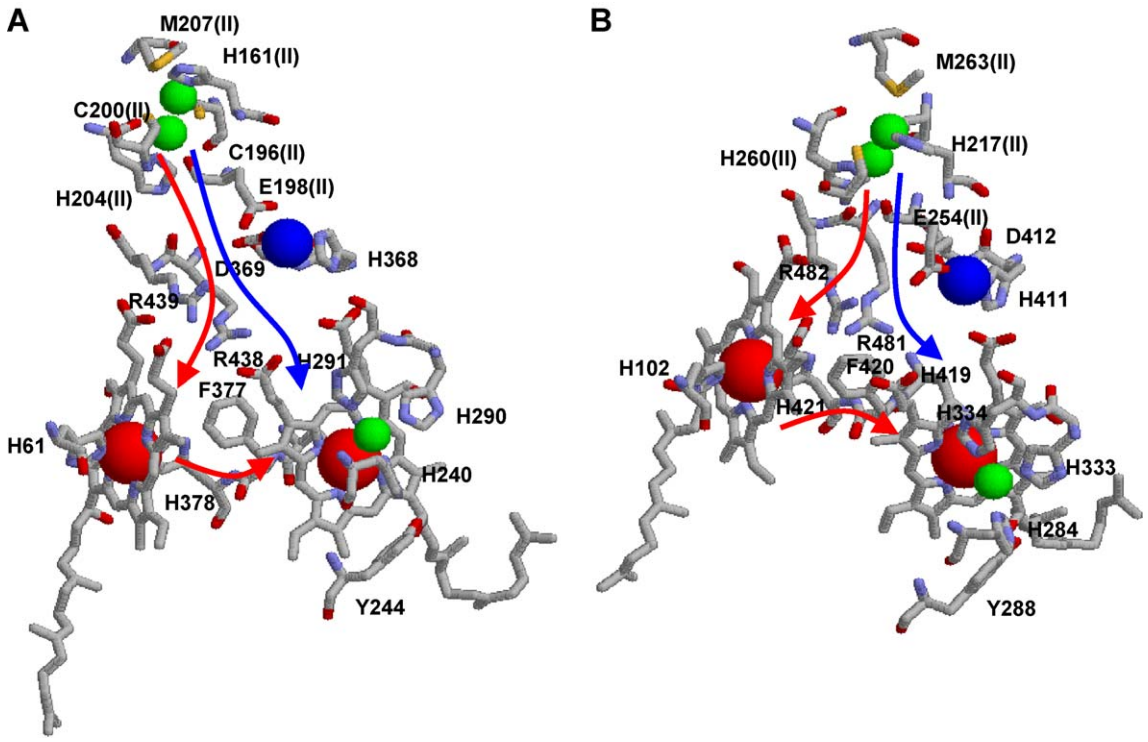

Fig. 4. View parallel to the membrane of the location in subunit I and II of bovine heart (A) and *R. sphaeroides* (B) of the metal centers and residues contributing to electron transfer pathways. The structures are drawn with Rasmol 2.7 from the PDB coordinate files of bovine heart (A, file 1V54) and *R. sphaeroides* (B, file 1M56) cytochrome *c* oxidase. Proton coupled transfer steps are shown by red arrows, decoupled electron transfer by blue arrows.

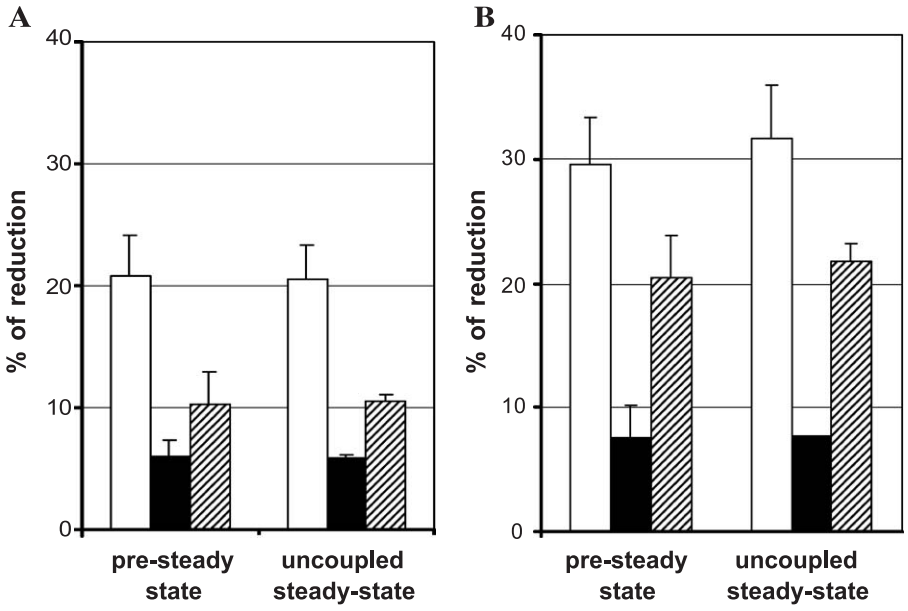

Fig. 5. Reduction levels of cytochrome *c* (empty bars), Cu_A (black bars) and heme *a* (dashed bars) measured in bovine heart cytochrome *c* oxidase vesicles under level flow (pre-steady state) and uncoupled steady-state conditions. The reduction levels were measured spectrophotometrically and are expressed as percentages of the full reduction attained for each metal redox center after addition of sodium dithionite. The reaction was initiated by the addition of stoicheiometric (A) or excess (B) cytochrome *c* with respect to the oxidase (in the presence of ascorbate and TMPD) to achieve low and high electron pressures, respectively. Valinomycin was present in the reaction medium. The pre-steady state reduction levels were obtained from the deconvolution of the time course of the reduction of the indicated metal centers; these levels were not affected by the presence of CCCP. The reduction levels of cytochrome *c*, Cu_A and heme *a* were followed by dual wavelength spectrophotometry at 550–540, 825–740 and 605–630 nm, respectively. To reduce the noise to signal ratio, in particular for Cu_A absorbance changes, the COV concentration was raised to 2 μM and the spectral contribution of cytochrome *c* was taken in account (see Ref. [39]).The steady state was achieved in the presence of a saturating concentration of CCCP. For experimental details, see Ref. [39]. Redrawn from Ref. [39].

to methionine, indicate that in the mutated oxidase electrons donated by cytochrome *c* can, at least in part, be directly transferred from Cu_A to the binuclear a_3–Cu_B center, thus bypassing heme *a* [40] (L.L. Palese, B. Ludwig, O.M.H. Richter, N. Capitanio, S. Papa, unpublished observations). Arg 54 is hydrogen-bonded to the formyl at position 8 of the porphyrin of heme *a* [40]. The R54M mutation decreases the E_m of heme *a* by more than 200 mV, resulting in a strong depression of electron transfer from Cu_A to heme *a*. As a result of this, the turnover of the oxidase is decreased to a small percentage of that exhibited by the wild-type enzyme [40]. Anaerobic reduction of the purified R54M oxidase by ascorbate plus cytochrome *c* shows that full reduction of cytochrome *c*, Cu_A and heme a_3 was reached while no reduction of heme *a* was detectable. Under these conditions, aerobic oxidation of the binuclear center was associated with synchronous oxidation of Cu_A (L.L. Palese, B. Ludwig, O.M.H. Richter, N. Capitanio, S. Papa, unpublished observations).

An extensive study of the influence of kinetic and thermodynamic factors on the H^+/e^- stoicheiometry of proton pumping of cytochrome *c* oxidase has been carried out by Papa et al. [23,39,41]. Measurements based on the determination of the initial rates of electron flow and proton ejection at level flow have shown that the H^+/e^- ratio of proton pumping, both in intact rat liver mitochondria [41] and in the isolated reconstituted bovine heart oxidase in phospholipid vesicles [39], sharply decreases from around 1 to around zero as the rate of electron flow is raised above a critical value. A similar decline in the H^+/e^- ratio of proton pumping associated with increase in the rate of electron flow was observed in reconstituted wild-type *P. denitrificans* cytochrome oxidase (L.L. Palese, B. Ludwig, O.M.H. Richter, N. Capitanio, S. Papa, unpublished observations). In addition, it was found that in the R54M mutant oxidase of *P. denitrificans* the H^+/e^- ratio, which was not higher than 0.25 at low electron transfer rates, decreased to zero as the rate of electron transfer was slightly enhanced (L.L. Palese, B. Ludwig, O.M.H. Richter, N. Capitanio, S. Papa, unpublished observations).

Proton pumping in cytochrome *c* oxidase has been found to be, at the steady state, partially decoupled by the ΔpH component of the PMF [39]. The situation is illustrated by the results presented in Fig. 6. It is shown here that at the respiring steady state in the presence of valinomycin, which converts all the PMF into a transmembrane ΔpH, the proton pumping activity of COV is practically fully depressed. By inducing a moderate proton leak with the addition of low concentrations of the protonophoric uncoupler CCCP, the ΔpH can be progressively decreased and this results in the progressive reappearance of a proton pumping activity (Fig. 6). This pattern is typical of the mutual relationship of leaks and slips in coupling membranes. Dissipation of PMF by leaks alleviates PMF-induced slip (decoupling) of proton pumps [42].

Putting together the functional and structural information available, it seems possible to propose that electron transfer

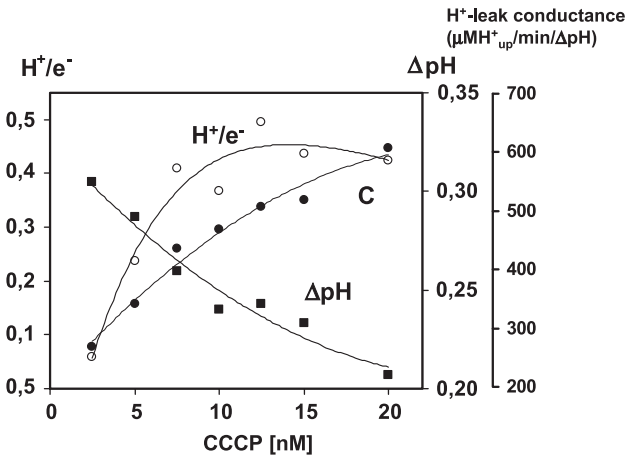

Fig. 6. Effect of ΔpH and proton leak on the steady-state H^+/e^- (empty circles) ratios in bovine heart cytochrome *c* oxidase vesicles. Membrane proton-leak conductance (black circles) and ΔpH (black squares) were modulated by additions of sub-saturating concentrations of CCCP in the presence of valinomycin. Respiration in valinomycin-supplemented COV was sustained by duroquinol oxidation, mediated by a trace of purified soluble bc_1 and cytochrome *c*, until a steady state was reached for redox-linked acidification of the medium and alkalinization of the vesicle interior. For other experimental details, see Ref. [39]. Reproduced with permission from Ref. [39].

from Cu_A to the heme a_3–Cu_B center can take place along two pathways. The first via heme *a* is associated with proton pumping with involvement of the proton/electron cooperative coupling at this heme. This would be the reason why nature has provided the oxidase with the low potential heme as an intermediate redox metal on the way to the binuclear center. The second pathway would consist of direct electron transfer from Cu_A to the binuclear center, bypassing heme *a* and thus resulting in decoupling of the proton pump. The actual H^+/e^- in the oxidase would be determined by the relative contribution of the two electron transfer pathways [23,39,41]. The relative contributions of the two pathways can be dictated by kinetic and thermodynamic factors. Under level flow conditions, the H^+/e^- is only influenced by kinetic factors. As the electron pressure and the rate of electron flow are increased above a critical threshold, the uncoupled electron transfer pathway, directly from Cu_A to the binuclear center, can become more important with a marked decrease in the H^+/e^- stoicheiometry. An alternative way in which kinetic factors can influence the efficiency of the proton pump is represented by the fact that its operation requires the system to alternate between an H^+ input (H^+ being taken up by critical protolytic residues from the N phase) and an H^+ output state [16,43]. When the rate of electron flow in the oxidase increases above a critical level, the speed at which the input–output states alternate can become inadequate to cope with that of electron flow, with loss of the asymmetry of protonation–deprotonation and decoupling of the pump.

The other factor controlling the contribution of the two electron transfer pathways is given by the PMF. ΔpH can

exert an inhibitory back-pressure on the proton coupled electron transfer pathway mediated by heme *a* without, obviously, affecting the uncoupled electron transfer pathway, thus enhancing the contribution of the latter with consequent decrease of the H^+/e^- stoicheiometry.

Decoupling of the oxidase at high electron pressure and high PMF can contribute to prevent excessive electronegativity of redox carriers in complex I and III, which, above a threshold level, can lead to production of deleterious oxygen radicals. Enhanced oxidase activity will prevent accumulation of toxic $O_2.^-$ and other free radicals deriving from it, also maintaining the cellular oxygen tension low [44,45].

4. Cooperative mechanisms in the proton pumps

In the light of data presented and a number of related results from various laboratories, variants for cooperative mechanisms in the proton pump of cytochrome *c* oxidase will now be examined.

The compulsory role of electron transfer via heme *a* in the proton pump of the cytochrome *c* oxidase seems to be generally accepted [22–24,27,39,46,47]. The hydrogen bond of R38 with the formyl substituent of heme *a* adjusts its E_m to a relatively high value so as to favour electron flow from Cu_A to the heme *a*. X-ray crystallographic analysis of various cytochrome *c* oxidases shows conserved structures which can provide fast electron transfer from heme *a* to heme a_3. [35–38]. The proton/electron coupling exhibited by heme *a* is likely to provide the basis for its obligatory role in the proton pump. Yoshikawa [15] and Tsukihara et al. [25] have described in detail a proton pumping mechanism based on proton/electron coupling at heme *a*, which excludes the O_2 reduction site (Fig. 7). In this mechanism, proton access from the N space to the environment of heme *a* is provided by a pathway, channel H, which is evident in the X-ray structure of the bovine [36] as well as *P. denitrificans* oxidase [35] but not so in the X-ray structures of other prokaryotic oxidases. The H channel is structurally well separated from the proton input channels D and K, which can be traced in the crystal structures of the oxidases described above [35–38]. The proton exit from the environment of heme *a* is proposed by Yoshikawa et al. [15,24,25] to be provided by a pathway involving R38, which is in fact conceived to represent an element of the gate of the pump, bound H_2O in the environment of heme *a*, one propionate of heme *a*, the peptide bond between Y440 and S441 and D51 at the P surface (Fig. 7). This exit pathway can be clearly traced in the bovine enzyme but not so in prokaryotic oxidases [48]. D51 buried inside the protein in the crystal structure of the fully oxidized bovine heart COX is moved by about 4.5 Å towards the P surface in the crystal of the reduced COX [24]. This is likely to result in a significant drop of the p*K* of the carboxylic group. Tsukihara et al. [25] have recently reported that site-specific

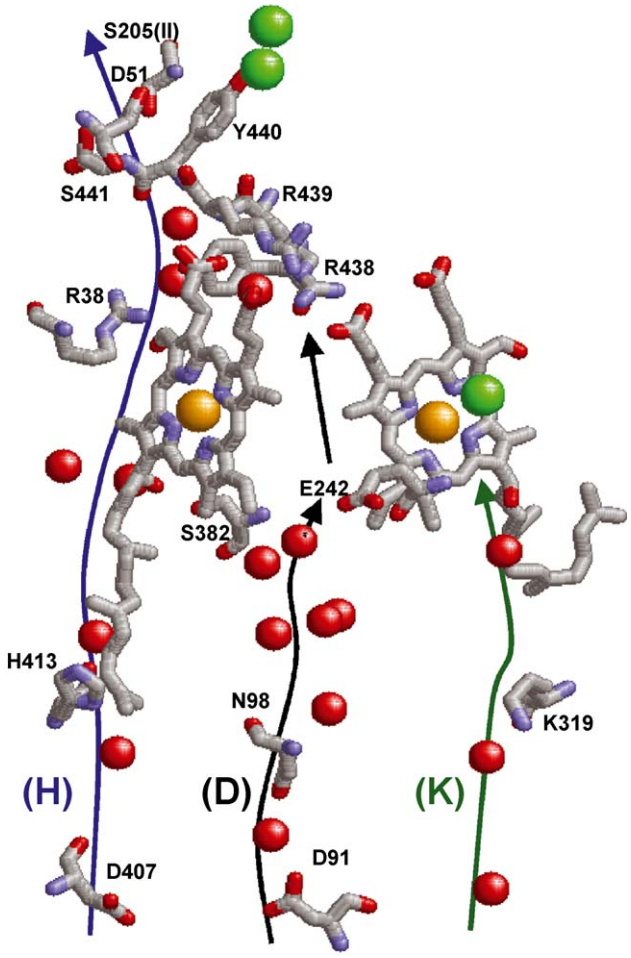

Fig. 7. View parallel to the membrane of the location in subunit I of cytochrome *c* oxidase of acid/base residues contributing to proton conducting pathways. The structure was drawn with Rasmol 2.7 from the PDB coordinates of the crystal structure of the fully oxidised bovine heart enzyme (1.8 A resolution, file 1V54). The red spheres show the position of water molecules intercalating protolytic residues along channels "H" (blue arrow), "D" (black arrows), and "K" (green arrow). See text for other details.

D51N mutation in the bovine COX abolishes proton pumping without impairment of the electron transfer activity.

On the basis of observations made in the CO-inhibited COX, Papa et al. [26,29,30] have developed a version of the Yoshikawa model, which utilises sharing of the proton/electron coupling of heme *a* and Cu_A with a common acid/base network. This version also makes use of the D51 proton exit pathway but leaves open whether the H or the D channel is used for proton input from the N space [23,26]. Related to this point is the observation that in *P. denitrificans* [49] and *R. sphaeroides* cytochrome *c* oxidase [50], mutation of asparagine 98 to aspartate, in the vicinity of D91 at the entry mouth at the N side of the D channel, abolishes proton pumping without impairment of electron transfer. Would this be in favour of the D channel? Or is it alternatively conceivable that the N98D mutation does impair proton/electron coupling at heme *a*? It should be

noted that the Yoshikawa model [15,25] as well as our version [26] both would, in principle, imply that transfer of each electron from heme *a* to the binuclear site is coupled to pumping of $\approx 1\ H^+$ from the N to the P phase.

Cooperative proton/electron coupling as the driving element of the proton pump and pathways of pumped protons well separated from those used by substrate protons will clearly prevent annihilation of pumped protons in the reduction of O_2 to H_2O. A difficulty in accepting the proton input H pathway and the D51 output pathway is raised, in addition to their poor conservation in prokaryotic oxidases by the lack of an effect on proton pumping of mutational replacement of candidate protonmotive residues in these pathways [48]. It can, however, be recalled that extensive amino acid sequence comparison and structural alignment of a large number of protonmotive heme-copper oxidases, as well as site-directed mutagenesis studies, show that not only in the H pathway but also in the D pathway candidate acid/base residues are not conserved; neither are they essential [18]. On the other hand, cavities are seen in these proton pathways which are or can be occupied by water molecules [25,37]. This water can contribute efficient proton transfer. Proton conduction pathways might, in fact, require a less stringent amino acid specificity than electron transfer pathways.

It is widely thought by other groups that the driving element of the pump is the heme a_3–Cu_B oxygen reduction site, where the proton pump is directly associated with the oxygen reduction steps [16,21,27,51–60]. It can, however, be noted that also in this case protonmotive interaction between oxido-reduction of the redox centers and acid/base residues in the apoprotein is envisaged [16,27,60]. It is generally accepted that the D pathway is used for the uptake from the N space of the third and fourth proton consumed in the reduction of O_2 to $2H_2O$, different from the first two protons which are taken up from the N space by a separate K channel [51–57]. Evidence has been provided indicating that at least in prokaryotic cytochrome *c* oxidase, the D pathway is also used for translocation of pumped protons [16,21,27,52,58]. Use of the D pathway for the transfer of both pumped and substrate protons requires a switch in the pathway between the two functions [16,23,59].Various observations indicate that such a switch can be performed by E242, located at the inner end of the D channel [16]. This residue would acquire two positions/states during the catalytic cycle [16,23,59,60]: one could be involved in proton pumping, the other in the translocation of protons consumed in the reduction of O_2 to H_2O. To explain the decoupling caused by the N98D mutation in the *R. sphaeroides* oxidase, it has been proposed that this mutation specifically affects the involvement of E242 in the translocation of pumped protons (possibly by increase of the p*K* of this residue) [60]. Evidence has, on the other hand, been produced indicating that the redox state of heme *a* [59], or of heme *b* in the bo_3 oxidase of *E. coli* [61], affects the (protonation) state of the E242.

A cooperative version of the pump might be conceived in which oxido-reduction of heme *a* (or *b*) determines the position/state of E242 in which it translocates pumped protons. Protons taken up by E242 upon reduction of heme *a* will then be transferred to the exit pathway upon electron transfer from heme *a* to the binuclear oxygen reduction site. Work from various laboratories indicates the involvement of propionates of heme a_3 [27,62] (see, however, Ref. [25]) and heme *a* [27] in the exit pathway of the pump. From the propionates protons could be transferred, via conserved arginines 438 and 439 [62], to the D51 segment and from this released in the P phase. This version of the pump would explain why both N98D and D51N mutations result in decoupling of the proton pump. It can also involve protonmotive interaction of the Cu_A/heme *a* with the heme a_3/Cu_B site. Involvement in the pump of the oxygen reduction site would open the problem of the steps of the catalytic cycle which are coupled to proton pumping. Mechanisms of direct involvement of the oxygen reduction catalysis have, in fact, originated an ongoing controversy as to which step of the catalytic cycle is coupled to proton pumping [27,54,63–65].

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